FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **112843-036**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): 10/018492

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/05403	**9 June 2000**	**11 June 1999**

TITLE OF INVENTION
BACTERIAL PROTECTION

APPLICANT(S) FOR DO/EO/US
Schmidt et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). unexecuted.
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Return Receipt Postcard

531 Rec'd PCT/ 07 DEC 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/018492	PCT/EP00/05403	112843-036

24. The following fees are submitted:.	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	15 - 20 =	0	x $18.00	**$0.00**
Independent claims	3 - 3 =	0	x $84.00	**$0.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**
TOTAL OF ABOVE CALCULATIONS =				**$890.00**
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**
SUBTOTAL =				**$890.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**
TOTAL NATIONAL FEE =				**$890.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**
TOTAL FEES ENCLOSED =				**$890.00**
				Amount to be: refunded $
				charged $

a. ☒ A check in the amount of **$890.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-1818** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Robert M. Barrett, Esq.
Bell, Boyd & Lloyd LLC
P.O. Box 1135
Chicago, Illinois 60690-1135
Tel: (312) 807-4204

SIGNATURE

Robert M. Barrett
NAME

30,142
REGISTRATION NUMBER

December 7, 2001
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Schmidt et al.
Appl. No.: PCT/EP00/05403
Filed: Filed Herewith
Title: BACTERIAL PROTECTION
Art Unit: Unknown
Examiner: Unknown
Docket No.: 112843-036

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified patent application as follows:

In the Claims:

Please amend Claim 1 as follows:

1. A method for protecting Lactobacillus johnsonii La1 against stress, which comprises the steps of treating Lactobacillus johnsonii La1 with a sublethal level of stress selected from the group consisting of thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

Please amend Claim 2 as follows:

2. The method of claim 1, which comprises the steps of treating with about 3.5% NaCl for 15 minutes.

Please amend Claim 3 as follows:

3. The method of claim 1, which comprises the steps of treating at a temperature of about 48°C for about 15 minutes.

Please amend Claim 4 as follows:

4. A Lactobacillus johnsonni La1 obtained through a process of subjecting Lactobacillus johnsonii La1 to a sublethal level of stress selected from the group consisting of thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

Please add Claims 5-15 as follows:

5. The method of claim 1 including the step of subjecting the Lactobacillus johnsonii La1 to a salt.

6. The method of claim 1 wherein the Lactobacillus johnsonni La1 is subjected to the stress for at least 15 minutes.

7. The Lactobacillus johnsonni La1 of claim 4, obtained through a process that comprises the steps of treating with about 3.5% NaCl for 15 minutes.

8. The Lactobacillus johnsonni La1 of claim 4, obtained through a process that comprises the steps of treating at a temperature of about 48°C for about 15 minutes.

9. The Lactobacillus johnsonni La1 of claim 4, obtained through a process that includes the step of subjecting the Lactobacillus johnsonii La1 to a salt.

10. The Lactobacillus johnsonni La1 of claim 4, wherein the Lactobacillus johnsonni La1 is subjected to the stress for at least 15 minutes.

11. A Lactobacillus johnsonni La1 that is protected against heat stress of up to 55°C for up to an hour obtained through a process comprising the step of subjecting Lactobacillus johnsonii La1 to a sublethal level of stress selected from the group consisting of thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

12. The Lactobacillus johnsonni La1 of claim 11, which comprises the steps of treating with about 3.5% NaCl for 15 minutes.

13. The Lactobacillus johnsonii La1 of claim 11, which comprises the steps of treating at a temperature of about 48°C for about 15 minutes.

14. The Lactobacillus johnsonii La1 of claim 11 including the step of subjecting the Lactobacillus johnsonii La1 to a salt.

15. The Lactobacillus johnsonii La1 of claim 11 wherein the Lactobacillus johnsonni La1 is subjected to the stress for at least 15 minutes.

REMARKS

This Preliminary Amendment is submitted in the above-identified patent application. Pursuant to the Preliminary Amendment, Claims 1-4 have been amended and newly-submitted Claims 5-15 have been added. This Preliminary Amendment does not add new matter.

Applicants also note for the record that this Preliminary Amendment is being submitted to place the claims in proper U.S. format and/or to add additional claims. Accordingly, Applicants submit that this Preliminary Amendment does not narrow the scope of protection being sought and therefore Applicants do not intend to disclaim any subject matter in view of this Preliminary Amendment.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Versions with Markings to Show Changes Made.**"

Respectfully submitted,

BELL, BOYD & LLOYD LLC

BY _____

Robert M. Barrett
Reg. No. 30,142
P.O. Box 1135
Chicago, Illinois 60690-1135
Phone: (312) 807-4204

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

1. (Amended) A method for protecting Lactobacillus johnsonii La1 against stress, which comprises the steps of treating [said micro-organism] Lactobacillus johnsonii La1 with a sublethal level of stress selected from the group[, which comprises] consisting of thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

2. (Amended) The method of claim 1, which comprises the steps of treating with about [3,5] 3.5% NaCl for 15 minutes.

3. (Amended) The method [according to] of claim 1, which comprises the steps of treating at a temperature of about 48°C for about 15 minutes.

4. (Amended) A Lactobacillus johnsonni La1 obtained [according to a method according to any of the preceding claims] through a process of subjecting Lactobacillus johnsonii La1 to a sublethal level of stress selected from the group consisting of thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

Please add Claims 5-15 as follows:

5. The method of claim 1 including the step of subjecting the Lactobacillus johnsonii La1 to a salt.

6. The method of claim 1 wherein the Lactobacillus johnsonni La1 is subjected to the stress for at least 15 minutes.

7. The Lactobacillus johnsonni La1 of claim 4, obtained through a process that comprises the steps of treating with about 3.5% NaCl for 15 minutes.

8. The Lactobacillus johnsonni La1 of claim 4, obtained through a process that comprises the steps of treating at a temperature of about 48°C for about 15 minutes.

9. The Lactobacillus johnsonni La1 of claim 4, obtained through a process that includes the step of subjecting the Lactobacillus johnsonii La1 to a salt.

10. The Lactobacillus johnsonni La1 of claim 4, wherein the Lactobacillus johnsonni La1 is subjected to the stress for at least 15 minutes.

11. A Lactobacillus johnsonni La1 that is protected against heat stress of up to 55°C for up to an hour obtained through a process comprising the step of subjecting Lactobacillus johnsonii La1 to a sublethal level of stress selected from the group consisting of thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

12. The Lactobacillus johnsonni La1 of claim 11, which comprises the steps of treating with about 3.5% NaCl for 15 minutes.

13. The Lactobacillus johnsonii La1 of claim 11, which comprises the steps of treating at a temperature of about 48°C for about 15 minutes.

14. The Lactobacillus johnsonii La1 of claim 11 including the step of subjecting the Lactobacillus johnsonii La1 to a salt.

15. The Lactobacillus johnsonii La1 of claim 11 wherein the Lactobacillus johnsonni La1 is subjected to the stress for at least 15 minutes.

Bacterial Protection

The present invention relates to a bacterial cell having protection against stress including the affects of extreme temperature change and osmotic shock; a nutritive or medicinal composition comprising the protected bacterial cell; and a method of protecting bacteria against stress.

Within the context of this specification the word "comprises" is taken to mean "includes, among other things". It is not intended to be construed as "consists of only". In addition, the word "stress" is used interchangeably with the term "adverse conditions". It includes, but is not limited to, adverse conditions of temperature (heat shock, cold shock), salt (osmotic shock), pH (pH shock), chemical stresses (antibiotics, alcohol, H2O2, etc.), nutritional stress, UV-stress, cold stress and oxygen concentration (oxidative stress).

Standard amino acid, RNA and DNA codes are used within this specification which are defined by the IUB Biochemical Nomenclature Commission.

It is well known that bacteria such as lactic acid bacteria (LAB) are ubiquitously found in the environment and they are largely used for the production of fermented products. For example, in the food industry bacteria are used in fermentation of milk products and production of starter cultures. During production of starter cultures, food fermentation, manufacture and storage, the bacteria that are employed must deal with different kinds of adverse conditions which generally have the effect of dramatically reducing their viability, stability and activity. These adverse conditions vary with production requirements and include thermal shock (freeze-drying or spray-drying), osmotic shock (drying) and pH shock (fermentation). It will be appreciated that the susceptibility or inability of bacteria to cope with these stresses is a problem in cases where bacteria are used on a large scale.

The presence of Bifidobacteria or lactobacilli in the human intestine, primarily the small and large intestine, is generally accepted as a contributing factor for a healthy well-being. In addition, it is considered that Bifidobacteria and lactobacilli may be useful in prophylaxis or treatment of ailments including

gastrointestinal infections. In the light of this, it has been suggested that large populations of Bifidobacteria and lactobacilli in the intestine should be maintained and products comprising the bacteria should be administered. Often these products comprise different species of Bifidobacteria or lactobacilli. However, the stresses that Bifidobacteria and lactobacilli are exposed to during manufacture and storage of the products can significantly reduce their viability and/or physiological activity.

The natural response by bacterial cultures to sublethal temperature shifts or other sublethal stresses (including exposure to oxygen and osmotic shock) includes rapid expression of a distinct set of polypeptides called "stress-proteins". These proteins have been shown to enable Gram-positive bacteria such as for example *Lactococcus lactis*, *Bacillus subtilis*, *Lactobacillus acidophilus*, *Lactobacillus sakei*, *Enterococcus faecalis*, and *Lactobacillus johnsonii* to adapt to otherwise growth-limiting conditions.

One of the most studied stress proteins are the heat shock proteins or chaperones. These proteins are generally involved in the maturation of newly synthesised proteins, and they assist in refolding of denatured proteins. Numerous stress-response genes have been characterised in LAB, including those encoding the two major chaperone machines (groES/groEL and hrcA/grpE/dnaK/dnaJ) involved in the proper folding of newly synthesised proteins and the repair of those that are denatured.

Remarkably, it has now been found that bacteria, including Bifidobacteria and lactobacilli, can be protected against levels of stress that are lethal in unprotected bacteria. Surprisingly, this can be done by subjecting the bacteria to a sublethal level of stress treatment. It has surprisingly been found that after this initial stress treatment a higher level of stress is required to adversely affect the bacteria. This is unexpected because it was thought that cells which are damaged by stress would be less likely to cope with additional stress. In fact, the converse has been found – pre-stressed cells are able to bear a higher stress level compared to control cells which have not been pre-stressed.

Protection against one form of stress acquired by treatment with a dissimilar form of stress has been referred to as "cross-protection". This is unexpected

because it was thought that cells damaged by treatment with one stress should render them more sensitive against an additional sublethal or lethal stress.

Accordingly, in a first aspect the invention provides a bacterial cell having protection against conditions which are lethal to an unprotected bacterial cell wherein, the protected cell is obtained by subjecting a bacterial cell to treatment with a sublethal level of stress.

In a second aspect, the invention provides a nutritive composition which comprises bacteria having protection against conditions which are lethal to unprotected bacteria wherein, the protected bacteria are obtained by subjecting bacteria to treatment with a sublethal level of stress and allowing them to recover.

In a further aspect, the invention provides a method of protecting a bacterial cell against stress which comprises the steps of treating a bacterial cell with a sublethal level of stress selected from the group which comprises thermal shock, osmotic shock, pH shock, oxidative stress, chemical stress, nutritional stress, UV-stress, cold stress.

Preferably the method includes the additional step of allowing the cell to recover.

Preferably chemical stress is provided by treatment with antibiotics, alcohol or H_2O_2.

Preferably, the bacterial cell is selected from the group which comprises Bifidobacteria, lactic acid bacteria, enterococci, streptomyces, and bacilli.

More preferably, the bacterial cell is *Bifidobacterium longum, Bifidobacterium adolescentis*, *Bifidobacterium breve* or *Lactobacillus johnsonii*. An advantage provided by these bacteria is that they have the ability to rapidly acidify their substrate, therefore producing microbiologically safe products. In addition they contribute to a healthy well-being in humans and animals. Furthermore, they display a protective role against attack by enteric pathogens and are associated with anti-carcinogenic, anti-mutagenic and anti-tumorgenic activities. Without wishing to be bound by theory, recent reports suggest that they might act directly

in the intestinal tract through antimicrobial activity, indirectly through immunomodulation via intestinal cells or by modifying the function of the normal indigenous microflora.

Preferably bacteria, more preferably Bifidobacteria and lactobacilli, are treated with sublethal salt concentrations to protect them against otherwise lethal salt concentrations or the cells are treated with sublethal thermal stress to protect them against otherwise lethal temperatures. Furthermore, results show that treatment with salt (e.g. NaCl) protect these bacteria against lethal thermal stress or against lethal cycles of freeze-thawing. Accordingly, the invention alternatively includes the steps of treating cells with salt to protect against thermal stress or treating the cells with adverse temperature conditions to protect against salt stress.

Preferably the bacterial cells are selected from *Bifidobacterium longum*, *Bifidobacterium adolescentis* or *Lactobacillus johnsonii*. More preferably the bacterial cells are selected from *Bifidobacterium longum* NCC481, *Bifidobacterium adolescentis* NCC251 or *Lactobacillus johnsonii* La1.

Preferably, protection against lethal salt concentrations (eg of between 0.1% and 0.4%) is carried out by treatment with about 0.01 to about 0.1% salt for about 15 to about 60 min. Preferably the salt is bile salt.

Preferably protection against lethal thermal stress (eg of between about 50°C to about 60°C) is carried out by treatment at about 37°C to about 50°C for about 15 to about 60 min or by treatment with a salt concentration of between about 1% and about 4% for about 30 to about 60 min.

Preferably, protection against freeze-thawing (eg 1 to 10 cycles) is carried out by treatment of the cells with salt concentration of between 1% and 4%.

Preferably *Bifidobacterium longum* NCC481 cells are protected. More preferably, protection of *Bifidobacterium longum* NCC481 cells is carried out in the logarithmic phase of their growth cycle against lethal bile salt concentrations (eg of between about 0.2% and about 0.3% for 30 min) by subjecting the cells to about 0.1% bile salt for about 30 min before lethal challenge.

Preferably, protection of *Bifidobacterium longum* NCC481 cells is carried out in the stationary phase of their growth cycle against lethal bile salt concentrations (eg of about 0.075% and about 0.15% for about 30 min) by treatment of the cells with about 0.05% bile salt for about 30 min before lethal challenge.

Preferably, *Bifidobacterium adolescentis* NCC251 cells are protected. More preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the logarithmic phase of their growth cycle against lethal bile salt concentrations (eg of between about 0.3% and about 0.4% for about 30 min) by subjecting the cells to about 0.1% bile salt for about 30 min before lethal challenge.

Preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the stationary phase of their growth cycle against a lethal bile salt concentration (eg of about 0.15% for about 30 min) by subjecting the cells to about 0.1% bile salt for about 30 min before lethal challenge

Preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the stationary phase of their growth cycle against the otherwise lethal effect of (eg about 3 to about 4 cycles) freeze-thawing (about -80°C to about room temperature (preferably about 20°C to about 30°C, more preferably 25°C)) by subjecting the cells to about 2% of NaCl for about 1 h.

Preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the logarithmic phase of their growth cycle against an otherwise lethal temperature of 55°C for 20 min by treatment of the cells for about 30 min at about 45°C, about 15 min at about 47°C or for about 1 h with 1% or 2% NaCl.

Preferably *Lactobacillus johnsonii* La1 cells are protected. More preferably, protection of *Lactobacillus johnsonii* La1 cells is carried out in the logarithmic phase of their growth cycle against an otherwise lethal temperature of 55°C for up to 1h by treatment of the cells with about 3.5% NaCl for about 15 min or about 48°C for about 15 min.

Preferably, protection of *Lactobacillus johnsonii* La1 cells is carried out in the stationary phase of their growth cycle against an otherwise lethal temperature of 55°C for up to 1h by treatment of the cells with a temperature of about 48°C for about 15 min or with about 3.5% NaCl for about 15 min.

Embodiments of the invention will now be described in further detail with reference to the accompanying drawings in which:

Figure 1 shows results of a dot blot hybridization of RNA from cells of *Bifidobacterium longum* NCC481 and *Bifidobacterium adolescentis* NCC251 after 10 min exposure to different kinds of stress. Hybridization was performed using the specific probes GSR8 and GSR5 for NCC481 and NCC251, respectively.

Figure 2 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 at 55°C after different pre-inductions in the logarithmic phase. Cells were grown in MRS and cysteine at 37°C to an OD600 of between 0.4 and 0.7. Aliquots were taken and subjected for 15 min to 47°C, for 30 min to 45°C, or 1 h to 1.5% NaCl or 2% NaCl; the control remained at 37°C. The samples were shifted to 55°C and after 10 and 20 min the viable cell counts were determined.

Figure 3 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 after three and four cycles of freeze-thawing. Stationary phase cells were taken and subjected for 1 h to 2% NaCl, the control remained without salt addition. The samples were shifted to -80°C and thawed at room temperature. This cycle was repeated three and four times before the viable cell counts were determined.

Figure 4 shows a graph of survival of *Bifidobacterium longum* NCC481 under lethal bile salt conditions in the logarithmic phase. Cells were grown to an OD600 (optical density at 600nm) between 0.4 and 0.7 and subjected for 30 min to 0.1% Oxgall. The control remained without Oxgall addition. The samples were aliquoted and shifted to 0.2%, 0.25%, and 0.3% Oxgall for 30 min, and the viable cell counts were determined.

Figure 5 shows a graph of survival of *Bifidobacterium longum* NCC481 under lethal bile salt conditions in the stationary phase. Cells were subjected for 30 min to an 0.05% Oxgall-treatment. The control remained without any Oxgall addition. The samples were aliquoted and shifted to 0.075%, 0.1%, and 0.15% Oxgall for 30 min, and the viable cell counts were determined.

Figure 6 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 under lethal bile salt conditions in the logarithmic phase. Cells were grown to an OD600 (optical density at 600nm) between 0.4 and 0.7 and subjected for 30 min to an 0.1% Oxgall-treatment. The control remained without any Oxgall addition. The samples were aliquoted and shifted to 0.3% and 0.4% Oxgall for 30 min, and the viable cell counts were determined.

Figure 7 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 under lethal bile salt conditions in the stationary phase. Cells were subjected for 30 min to an 0.1% Oxgall-treatment. The control remained without any Oxgall addition. The samples were aliquoted and shifted to 0.15% Oxgall for 30 min, and the viable cell counts were determined.

Figure 8 shows a graph of survival of *Lactobacillus johnsonii* La1under lethal thermal conditions. Cells were grown in MRS at 37°C to an OD600 (optical density at 600nm) between 0.4 and 0.7. Samples were taken and subjected to 3.5% NaCl or 48°C for 15 min. The control remained at 37°C. Afterwards the samples were shifted to 55°C and the viable cell counts were determined after 30 min and 60 min.

Figure 9 shows a graph of survival of *Lactobacillus johnsonii* La1 in the stationary phase of their growth cycle under lethal thermal conditions. Samples were taken and subjected to 3.5% NaCl or 48°C for 15 min. The control remained at 37°C. Afterwards the samples were shifted to 55°C and the viable cell counts were determined after 60 min.

Strains and growth conditions

Bifidobacterium adolescentis NCC251, *Bifidobacterium longum* NCC481, *Bifidobacterium longum* NCC490, *Bifidobacterium longum* NCC585, and

Bifidobacterium breve NCC298 were cultivated in MRS medium supplemented with 0.5 g/l cysteine at 37°C under anaerobic conditions (98% nitrogen and 2% hydrogen). *Lactococcus lactis* MG1363 was grown in MRS medium at 30°C. *Escherichia coli* TG1 (Amersham) was cultivated in Luria-Bertani medium at 37°C. *Lactobacillus johnsonii* La1 was grown in MRS at 37°C.

Stress treatment

Cells were grown to an OD600 (optical density at 600nm) between 0.4 and 0.7 or taken in the stationary phase and subjected for different times to various stress conditions. Cells used for freeze-thawing experiments were concentrated in saline solution before being subjected to –80°C. Salt stress was exerted by adding sodium chloride to the samples while for bile-salt stress OXGALL (Trade Mark) (Difco) was used.

The stress treatment of Bifidobacteria was performed under anaerobic conditions while the determination of viable cells was carried out under aerobic conditions. Cells of lactobacilli were grown under microaerophil conditions, stress treatments and determination of viable cell counts was performed under aerobic conditions.

Bifidobacteria

Ranges for inductions and lethal challenges

	re-induction	lethal challenge
pH (e.g. HCl):	pH 6.0-3.5	pH 2.5-2
Bile (e.g. Oxgall):	0.01%-0.1%	0.075%-0.4%
Temperature:	37°C-48°C	50°C-60°C
Salt (e.g. NaCl):	0.5%-3%	3%-8%

Time of pre-induction and lethal challenge can vary dependent on strain and stress conditions between 5 min to 2 h.

Lactobacilli

Ranges for inductions and lethal challenges

	re-induction	lethal challenge
pH (e.g. HCl, lactic acid)	pH 6.0-4.5	pH 4.0-2
Temperature	40°C-50°C	50°C-60°
Salt (e.g. NaCl)	0.5%-3.5%	4%-8%

Time of pre-induction and lethal challenge can vary dependent on strain and stress conditions between 5 min to 2 h

DNA techniques

Isolation of chromosomal DNAs was carried out according to standard methods.

Analyses of mRNA

For Dot-blot hybridisation total RNA was isolated, denatured and transferred to uncharged nylon membranes (GeneScreen, NEN) according to standard methods. The membranes were pre-hybridised (1h, 40°C) and subsequently hybridised for 4h with 100 pmol DIG-labelled probes (Boehringer). The membranes were washed twice for 5 min in 2x SSC containing 0.1% SDS at 40°C and once at the probe-dependent temperature, which was 46°C and 48°C, respectively for the two dnaK specific probes GSR5 (5′-CATCGAAGGTGCCGCCAC-3′) and GSR8 (5′-TCGTCACCACCGAGGTG-3′), and 51°C for the universal probe 1028R (5′-CCTTCTCCCGAAGTTACGG-3′). Detection was performed according to the manufacturers instructions.

PCR amplification

The core dnaK region was amplified using the degenerate primers HS1 (5′-ATIACIGTICCIGCITA (T/C)TT(T/C)AA(T/C)GA-3′) and HS2 (5′-CATIGT(T/C)TCIATICCIA(A/G)IGAIA(G/A)IGG-3′) as well as 1 µg of chromosomal DNA as template. Amplification reactions were performed in a total volume of 100 µl (containing 200 µM each of dATP, dCTP, dGTP, and dTTP, 50 pmol of each primer, 2.5 U of Super-Taq DNA Polymerase (HT Biotechnology), and the corresponding 1x PCR buffer). Reactions were carried out with a Perkin-Elmer thermocycler: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Identification of the dnaK gene

Based on the alignment of Barril et al. (1994), we chose two regions of the DnaK of *Lactococcus lactis, Escherichia coli* and *Bacillus megaterium* possessing identical amino acid sequences and designed two degenerate primers HS1 and HS2 corresponding to the amino acids at positions 114 to 122 and 366 to 374 of *Lactococcus lactis* DnaK, respectively. This primer pair was used for a PCR-amplification using chromosomal DNA of NCC481, NCC490, NCC585, NCC251, and NCC298 as templates. Two fragments were obtained for each strain. For all strains those fragments corresponding in size to that of the two positive controls *Escherichia coli* and *Lactococcus lactis* were isolated from an agarose gel, purified and sequenced. In each fragment an open reading frame was identified showing high sequence similarities to the core region of known DnaK proteins. Particularly high identities were observed to streptomyces and mycobacteria as well as to *Lactobacillus sakei*, bacilli, and streptococci.

mRNA analysis of *dnaK* gene expression

The transcriptional induction of dnaK was investigated with cells exposed to heat shock and to additional general stress conditions. *Bifidobacterium longum* NCC481 and *Bifidobacterium adolescentis* NCC251 cells of the logarithmic phase were subjected to 0.1% bile salt, 1.5% NaCl or a heat shock for 10 min at 42°C and 45°C. Maximum temperatures of 47°C and 50°C were tested for NCC481 and NCC251, respectively. Uninduced cells from the logarithmic and stationary phase were always used as controls. Total RNA was isolated and subjected to dot blot hybridization. The dnaK specific probes GSR8 and GSR5 were used for NCC481 and NCC251, respectively. The universal probe 1028R was chosen to verify the amount and quality of RNA on the membrane. An increased concentration of dnaK specific mRNA was observed when subjecting the cells to increasing temperatures (Figure 1). In contrast to NCC251, dnaK of NCC481 was only slightly induced in cells entering the stationary phase. Furthermore a slight induction of dnaK was observed in NCC251 after bile-salt and NaCl treatment. No significant induction under identical conditions was obtained for NCC481.

Survival and cross-protection

Growth and survival of *Lactobacillus johnsonii* La1, *Bifidobacterium adolescentis* NCC251 and *Bifidobacterium longum* NCC481 at different temperature, bile-salts and salt conditions were tested.

Remarkably, logarithmic phase NCC251 showed an increased resistance to the generally lethal temperature of 55°C after being treated with sublethal heat stress. An almost 24-fold and 128–fold higher thermotolerance was observed after subjecting the cells to 47°C for 15 min prior to a heat shock for 10 min and 20 min, respectively (Figure 2). These figures are remarkable because they show how that, unexpectedly effective pre-induction of cells can be to protect them against otherwise lethal challenges. A 9-fold and 15-fold cross-protection of cells against 55°C was achieved by pretreatment for 1h with 1.5% NaCl. An equal protection against thermal stress could also be observed by pre-inducing at 45°C for 30 min or 2% NaCl for 1 h (Figure 2).

Cells in the logarithmic phase of the growth cycle of *Lactobacillus johnsonii* La1 showed a 400-fold higher protection against 55°C for 30 min after being pretreated with 3.5% NaCl or 15 min 48°C. After one hour at 55°C, a 10-fold and 5-fold higher protection was observed against 55°C in samples pretreated with 3.5% NaCl and 48°C for 15 min, respectively (Figure 8).

Stationary phase cells of *Lactobacillus johnsonii* La1 showed a remarkable 20-fold higher protection against 55°C for 1 hour after being treated with 3.5% NaCl for 15 minutes at 48°C (Figure 9).

Cells of *Bifidobacterium adolescentis* NCC251 in the stationary phase demonstrated a 14-fold higher survival after continuous cycles of freeze-thawing if pre-stressed with 2% NaCl for 1h (Figure 3). After 4 cycles of freeze thawing a 10-fold higher survival was observed.

Protection against lethal bile-salt concentrations could be observed in the logarithmic as well as in the stationary phase of *Bifidobacterium adolescentis* NCC251 and Bifidobacterium longum NCC481. A preconditioning (e.g. 30 min) of logarithmic cells with 0.1% bile-salts resulted in a 300-fold and 21-fold

protection against 0.3% and 0.4% bile-salts in logarithmic phase cells of *Bifidobacterium adolescentis* NCC251, respectively, (Figure 6). An 81-fold increased survival of stationary phase cells of *Bifidobacterium adolescentis* NCC251 (pre-induced with 0.1% bile-salts) was observed under the lethal concentration of 0.15% bile-salts (Figure 7). Analogous results were obtained for *Bifidobacterium longum* NCC481. Logarithmic cells, pre-induced with 0.1% Oxgall showed a 400-fold, 1800-fold, and 580-fold better survival against the lethal concentrations of 0.2%, 0.25%, and 0.3% Oxgall, respectively (Figure 4).Cells of the stationary phase showed a 3-fold, 29-fold, and 150-fold better survival for 30 min against 0.075%, 0.1%, and 0.15% Oxgall when they were pre-induced for 30 min with 0.05% Oxgall (Figure 5).

In contrast to the results published by Flahaut *et al.* (1996) where a protection of *Enterococcus faecalis* cells against 0.3% bile salts could only be achieved for 30 seconds, remarkably cells were able to be protected for 30 min against lethal bile salt concentration. This could not have been predicted.

The core region of dnaK of *Bifidobacterium longum* NCC481, *Bifidobacterium longum* NCC490, *Bifidobacterium longum* NCC585, *Bifidobacterium adolescentis* NCC251, and *Bifidobacterium breve* NCC298 were PCR-amplified and identified. Subsequent mRNA analyses revealed that in NCC251 and NCC481 the induction of dnaK is regulated at the transcriptional level. Transcription is generally induced by heat and for NCC251 also by treatment with salt and bile-salts.

In the light of these findings it has been concluded that stress pre-treatment of Bifidobacteria and/or lactobacilli can lead to a significantly increased chances of survival under otherwise lethal homologous or heterologous stress conditions.

Applicant: Société Des Produits Nestlé S.A.
Our file: 80295 WO

Claims

1. A method for protecting Lactobacillus johnsonii La1 against stress, which comprises the steps of treating said micro-organism with a sublethal level of stress selected from the group, which comprises thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

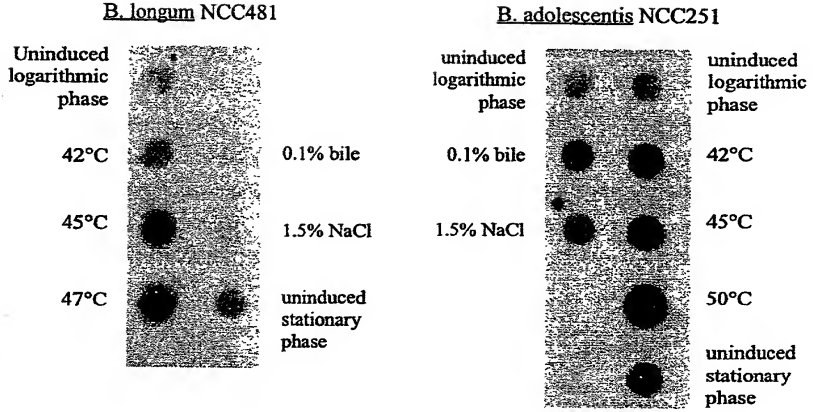
2. The method of claim 1, which comprises the steps of treating with about 3,5 % NaCl for 15 minutes.

3. The method according to claim 1, which comprises the steps of treating at a temperature of about 48 °C for about 15 minutes.

4. A Lactobacillus johnsonni La1 obtained according to a method according to any of the preceding claims.

10/018492

Figure 1



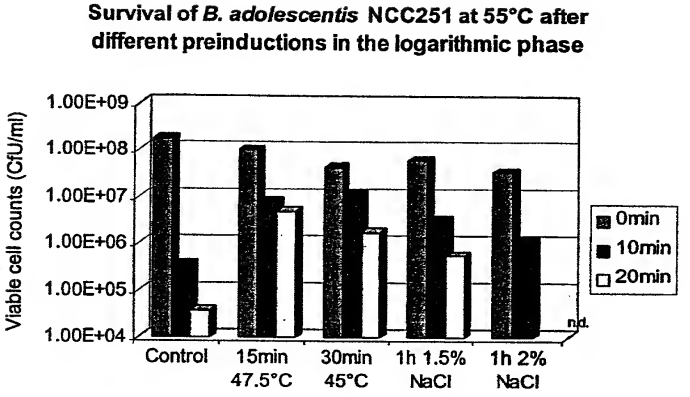
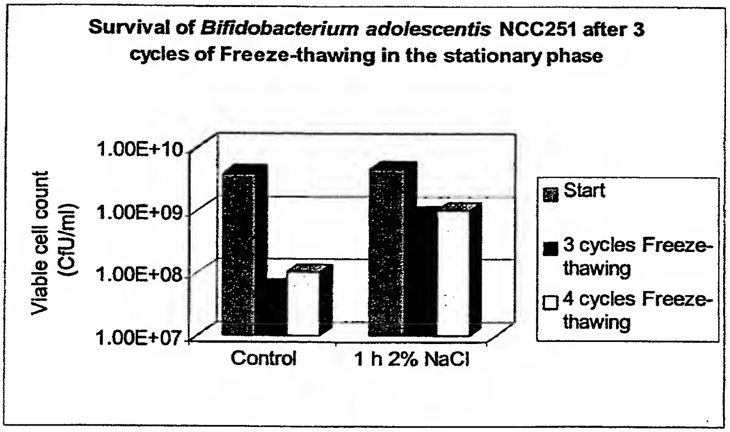
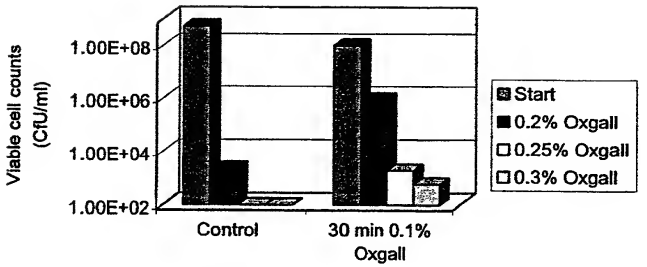


Figure 2

10/018492

Figure 3




Viable cell counts (CfU/ml)
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1.00E+06
1.00E+04
1.00E+02
Control
30 min 0.1% Oxgall
Start
0.2% Oxgall
0.25% Oxgall
0.3% Oxgall

Figure 4

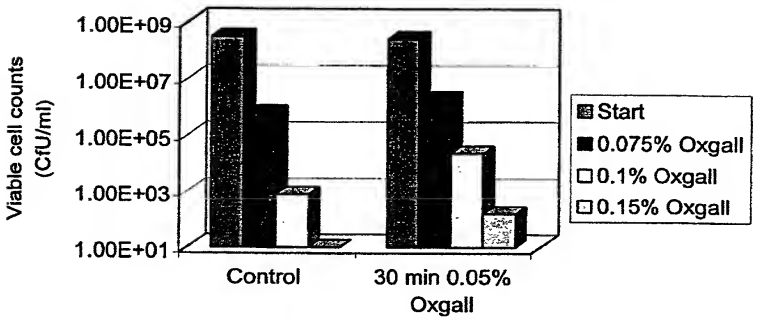

Figure 5

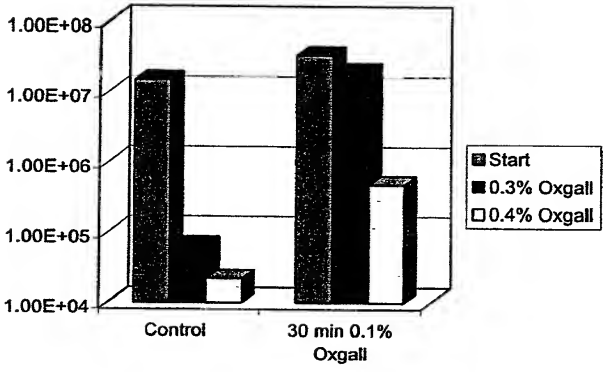
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Control
30 min 0.1% Oxgall
Start
0.3% Oxgall
0.4% Oxgall

Figure 6

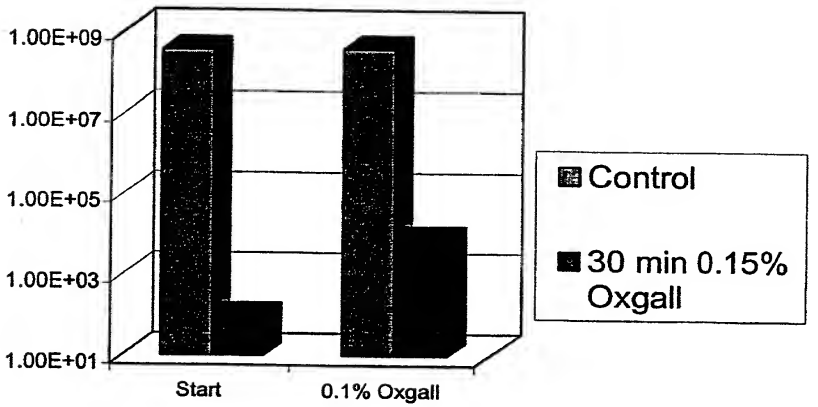

Figure 7

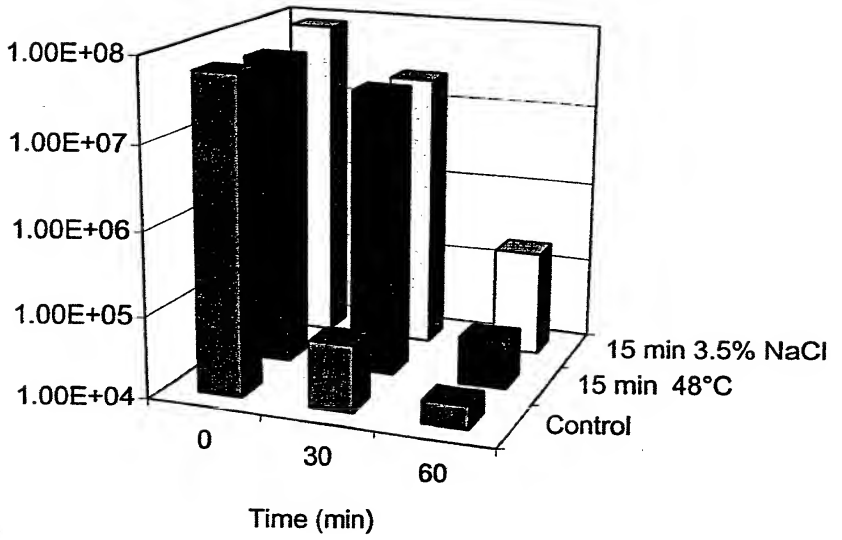

Figure 8

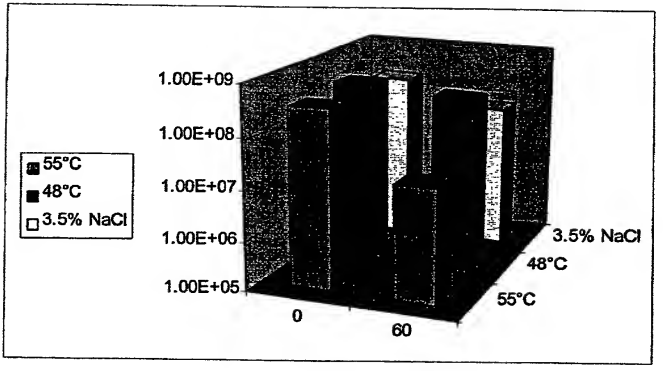

Figure 9

Docket No. 112843-36

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BACTERIAL PROTECTION

the specification of which: (check one)

☐ is attached hereto.

☒ was filed on June 9, 2000, as United States Application No. or PCT International Application No. PCT/EP00/05403 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent Office all information which is known to me to be material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code Section 119 or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Day/Month/Year Filed	Priority Not Claimed
			☐
			☐
			☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

Application Serial No.	Filing Date
60/138,946	June 11, 1999

I hereby claim the benefit under 35 U.S.C. Section 120 of any United States application(s), or Section 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C.F.R., Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint the practitioners at customer number: 29157

as my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and direct that all correspondence be forwarded to:

Bell, Boyd & Lloyd LLC
P.O. Box 1135
Chicago, Illinois 60690-1135

And all telephone calls be directed to: (312) 807-4204.

Docket No. 112843-36

Full name of sole or first inventor 1-00 **Gudrun Schmidt**	
Sole or first inventor's signature Gudrun Schmidt	Date 20.2.02
Residence ~~CH-1010 Lausanne, Switzerland~~ D-70327 STUTTGART DEX	
Citizenship Germany ✓	
Post Office Address ~~Chemin De Bérée 56~~ Widukindstrasse 11	
~~CH-1010 Lausanne, Switzerland~~ D-70327 STUTTGART, Germany	

Full name of second inventor 2-00 **Ralf Zink**	
Second inventor's signature Ralf Zink	Date 28/01/02
Residence CH-1801 Le Mont Pélerin, Switzerland CHX	
Citizenship Germany ✓	
Post Office Address Chemin de la Maison Jean 36	
CH-1801 Le Mont Pélerin, Switzerland	

Full name of third inventor	
Third inventor's signature	Date
Residence	
Citizenship	
Post Office Address	